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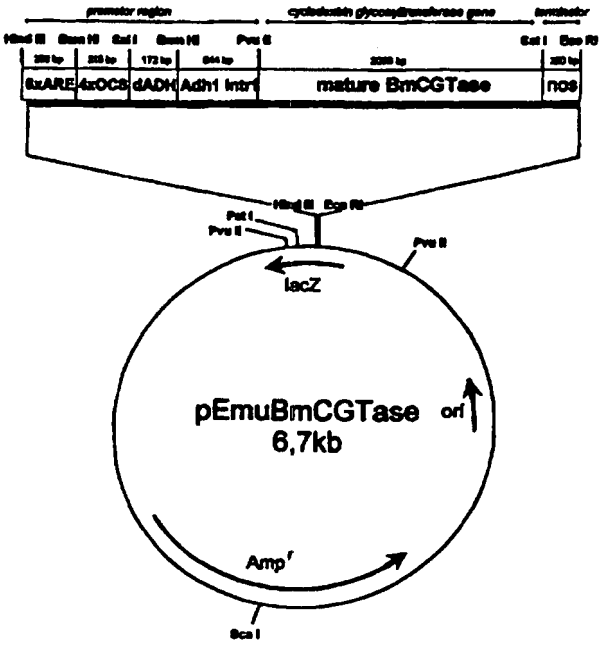
(57) Abstract

The invention describes a process for the production of degradation and/or conversion products of storage substances present in transgenic plant material with the help of malting processes as well as the resulting malted plant material and the malting solution.



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Process for the production of degradation and/or conversion products of storage substances present in transgenic plant material with the help of a malting process

The present invention relates to processes for the production of degradation and/or conversion products of storage substances present in transgenic plant material with the help of malting processes, as well as to the malted transgenic plant material and the malting solution produced by such processes.

The production of heterologous products, namely of heterologous polypeptides, in transgenic plants had gained increasing significance over the last years. Besides the overexpression of plant proteins or their reduction in transgenic plants in order to modify the plant metabolism and to obtain modified or improved products from plants, transgenic plants are also more and more used for the production of heterologous products normally not occurring in plants, for example bacterial toxins, mammalian proteins, biodegradable plastics or specific kinds of carbohydrates, such as saccharides and the like.

However, a drawback connected with the use of transgenic plants for the production of a desired product is that the conditions for expression of the introduced gene cannot be exactly regulated. In cases in which it is necessary to tightly control the expression of the introduced gene, often inducible promoters have to be used. The induction of such promoters by chemical or physical means in the field, however, requires high expenses of costs and work.

A different approach for controlling the expression of the introduced gene is disclosed in WO-95/14099 providing for the production of heterologuous proteins by malting of monocot plant seeds. The heterologous genes are expressed during germination of the seeds and the heterologous proteins are isolated from the malt.

The storage substances (starch, fat, etc.) contained in the plant material, are, however, not consumed in said malting process. For making the most of the resources of plant material it would thus be highly desirable to provide processes which would use these storage substances for producing other useful products from transgenic plants.

Hence, the present invention addresses the problem of providing processes which allow for the production of desired products from storage substances in transgenic plant tissue under conditions which may be easily controlled.

This object is achieved by the embodiments characterized in the claims.

Thus, the present invention relates to a process for the production of degradation and/or conversion products of storage substances present in transgenic plant material, which process comprises

(a) subjecting transgenic plant material to a malting process, wherein the transgenic plant material comprises at least one recombinant nucleic acid molecule containing

(i) at least one nucleic acid sequence coding for at least one heterologous polypeptide possessing enzymatic activity which leads to the degradation and/or conversion of a storage substance present in the transgenic plant material; and

(ii) regulatory elements which allow for the ex-

pression of said nucleic acid sequence(s) in the transgenic plant material during the malting process,

whereby said heterologous polypeptide(s) is (are) synthesized; and

(b) degrading and/or converting the storage substance present in the transgenic plant material by said heterologous polypeptide(s) in the malting solution.

By "malting process" a process is meant which comprises the germination of plant material under artificial or controlled environmental conditions. Conventionally such a process is carried out during the production of beer and comprises steps of steeping of the plant material that should be malted and subsequent germination under controlled conditions. By "controlled conditions" it is meant that physical parameters like humidity, temperature, salt concentrations, and agitation are controlled by human intervention. Generally this process may be essentially carried out as described, for example, "Die Bierbrauerei", Band 1: Die Technologie der Malzzubereitung, Ferdinand Enke Verlag Stuttgart. Depending on the plant material used the conditions employed in the malting process may vary. Malting processes are mainly described for the malting of seeds of corn plants, mainly of wheat and barley. However, also the malting of seeds of other plants, such as maize, rice, millet, rye, Triticale, manioc, soy bean as unmelted grain, sunflower or oilseed rape have been described (see, for example, "Handbuch für die Brauwirtschaft/Vergleichs-daten zur Betriebs- und Qualitätskontrolle", Alfa-Laval Industrietechnik GmbH, Hamburg). The malting processes described in the art may be modified in order to allow the malting of suitable material, namely of seeds, from other plants.

The product obtained by the malting process, in the case of

corn called "green malt", is a product especially rich in enzymes which are necessary for the conversion of storage substances. The malting process is a highly controlled process which allows a specific regulation of the production of enzymes in the malted material. Normally, the product obtained is subsequently kiln-dried leading to the so-called "cured malt".

By "transgenic plant material" it is meant that this plant material comprises transgenic plant cells which contain stably integrated into the genome at least one recombinant nucleic acid molecule which is either heterologous with respect to the transgenic plant material and normally not present in nontransformed cells or which is homologous with respect to the transgenic plant material but located in a different genomic environment in the transgenic cells in comparison to wildtype cells.

The transgenic plant material which is used in the process according to the invention may be any plant material which may be subjected to a malting process and contains a degradable and/or convertable storage substance. Preferably the plant material is a seed. The material may be derived from any suitable transgenic plant, in particular from any suitable monocotyledonous or dicotyledonous plants. Preferred are agricultural or horticultural plants, in particular plants which set seeds.

In a preferred embodiment the transgenic plant material is derived from a plant which is able to synthesize and store starch, such as corn or cereal plants or leguminous plants.

Preferred corn or cereal plants are those of the family Poaceae, such as wheat (Triticum), barley (Hordeum), rye (Secale), oats (Avena), rice (Oryza), maize (Zea), millet (Panicum, Setaria, Pennisetum or Sorghum) etc.

Preferred starch-storing leguminous plants are, for example those of the genus Vicia (e.g. V. faber), Pisum (e.g. P. sativum), Cicer (e.g. C. arietinum), Lens (e.g. L. culinarum) and Phaseolus (e.g. P. vulgaris or P. coccineus).

Accordingly, the heterologous polypeptide may be any polypeptide having an enzymatic activity which leads to the modification or conversion of starch in order to obtain a desired product. Examples of such enzymes are all kinds of amylases, glucanases, branching enzymes, debranching enzymes, disproportioning enzymes, starch phosphorylases, dextrinases, etc. DNA sequences coding for the above mentioned enzymes are well known in the art. Preferred polypeptides used in the process according to the invention are those which catalyse the conversion of starch, namely of amylose, to cyclodextrins. These enzymes are called cyclodextrin glycosyltransferases (CGTase; EC 2.4.1.19) and are found in a number of Bacillus species, for example, in Bacillus ohbensis (Sin et al., Appl. Microbiol. Biotechnol. 35 (1991),600-605) and Bacillus macerans (Takano et al., J. Bacteriol. 166 (1986), 1118-1122) and in Klebsiella pneumonia (Bender, Arch. Microbiol. 111 (1977), 271-282). Examples for DNA sequences coding for a cyclodextrin glycosyltransferase are those described in Takano et al. (loc. cit.), Binder et al. (Gene 47 (1986) 269-277) or Kimura et al. (Appl. Microbiol. Biotechnol. 26 (1987), 149-153). Further sequences are publicly available in data bases such as the EMBL data base. Cyclodextrins are cyclic oligosaccharides consisting of six, seven or eight α-1,4-linked glucose molecules. Due to their cyclic structure they form an apolar cavity with a hydrophilic exterior and can easily form inclusion complexes with hydrophobic substances. This renders them particularly useful for different purposes in pharmaceutical and industrial applications. Due to their properties they may be employed, for instance, in the clearing of sewage water, the encapsulation of vitamins, the removal of specific metabolites, such as cholesterol, from

body fluids, as catalysts in chemical reactions and the like.

With respect to the degradation or conversion of starch by the heterologous polypeptide(s) one advantage of the process according to the invention is, for example, that it is possible to bring the polypeptide in direct contact with the starch which allows a high efficiency of the enzymatic conversion. The polypeptide can, for instance, be specifically expressed in the aleurone layer of starch storing seeds of corn plants. This leads to secretion of the polypeptide by the aleurone cells upon germination and subsequent diffusion into the endosperm. Here the starch which makes up as much as 70% of the seed weight in some corn species is directly accessible to degradation and thereby allowing a highly efficient degradation and/or conversion of the starch.

The preferred transgenic plant material for producing cyclodextrins are wheat and barley seeds and potatoes.

In another preferred embodiment the transgenic plant material is derived from a plant which uses as a storage substance predominantly oil or fat, such as rapeseed, sunflower, soy bean (Glycine soja), pea nut (Arachis hypogaea), linseed (Linum usitatissimum), plants of the orders Euphorbia (e.g. Simmondsia species), Turnera, Vernonica or the like.

Accordingly, the heterologous polypeptide may be any polypeptide having an enzymatic activity which leads to the degradation or modification of oil, fats, fatty acids or lipids in the transgenic plant material. This may lead either to the synthesis of fatty acids or lipids having desired properties or to the conversion of fatty acids or lipids into other metabolic intermediates which may serve as starting material for the synthesis of other desired products. Examples of such enzymes are enzymes involved in the introduction of hydroxy or epoxy groups in fatty acids, enzymes which

modify the degree of desaturation or saturation of fatty acids, such as desaturases, e.g. stearoyl-ACP desaturase, enzymes that modify the length of fatty acids, acyltransferases, 3-ketoacyl-ACP synthetase, acetyl-CoA synthetase, lipases, decarboxylases, etc. The possibility of lipid modification or of the modification of the composition of lipids in plant seeds has been described, for example, in Ohlrogge (Plant Physiol. 104 (1994), 821-826). These possibilities include, for example, the degree of desaturation and the variation of length of fatty acids.

Moreover, such degradation and/or conversion reaction of oil, fats, etc. can be used to produce polyhydroxy fatty acids (PHF) (a summary on the production of polyesters in plants being given in F. R. van der Leij and B. Witholt, Can. J. Microbiol. 41 (Suppl. 1), 222-238 (1995)). A suitable plant material for this purpose is rapeseed and sunflower.

In a yet further preferred embodiment the transgenic material is derived from a plant which uses as a storage substance protein(s), such as soy beans or the above-defined starch-storing corn and leguminous plants which are also producing protein rich seeds. The storage proteins may belong, for example, to the group consisting of glutelines, prolamines, globulines and albumines.

Accordingly, the heterologous polypeptide may be a polypeptide catalysing the degradation of proteins, for example, proteases such as endo- or exoproteases or -peptidases.

The obtained degradation and/or conversion product is either an amino acid or a derivative thereof, an oligopeptide, or a polypeptide. Examples for such polypeptides are industrial and technical enzymes (for a long but not exhaustive list, see, for example, WO 92/01042) or any polypeptides which may be useful in pharmaceutical or diagnostic applications, such

as polypeptide hormones such as growth hormones, neuropeptides, growth factors, clotting factors, clotting inhibiting factors like protamines or the thrombin inhibitor huridin, proteinase inhibitors like antithrombin III, perforines, interferones, interleukines, colony stimulating factors, erythropoietin, antiviral or antibacterial proteins, lectins, proteins with tumor suppressor activity, such as ricin of the seeds of Ricinus, etc. (for the production of such biomolecules in transgenic plants see: O. J. M. Goddijn, J. Pen, TIBTECH, Sept. 1995 (Vol. 13), 379-387). It is also possible that a precursor protein is produced in a first step (first gene), which is subsequently cleaved or modified by a second enzyme (second gene), e.g., a protease or another protein-modifying enzyme, whereby both genes are induced by the malting process (see J. Vandekerckhove et al. in TIBTECH, Jan. 1990 (Vol. 8), p. 1 and Biotechnology, Vol. 7, p. 929 (1989)). Of particular interest is the synthesis of polypeptides which may be used for vaccination, such as specific antigens of viruses, bacteria or other pathogenes as, for example, protozoa (see "Transgenic plants as vaccine production systems", H. S. Mason, C. I. Arentzen, TIBTECH, Sept. 1995 (Vol. 13), p. 388).

Another possible application is the synthesis of antibodies, for example IgG, IgA, IgM, or fragments of antibodies like Fab fragments, V_H chains or single chain antibodies (for the production of antibodies in plants see J. K-C. Ma and M. B. Hein, TIBTECH, Dec. 1995 (Vol. 13), 522-527). Of special interest is the possibility of producing catalytic antibodies.

By "heterologous" with regard to the polypeptide which is synthesized in the transgenic plant material during the malting process is meant that such a polypeptide is encoded by a DNA molecule which either originates from a different organism, such as a bacterium, fungus, animal or another

plant having another genotype than the transformed plant or by a DNA sequence which originates from a plant displaying the same genotype as the transformed plant but which is not in the same genomic environment as is such a DNA sequence when it is naturally found in the organism from which it originates. This means that this polypeptide may be encoded, for example, by a DNA sequence which is endogenous to the transgenic plant cell and which is also under the control of the same regulatory elements as the endogenous counterpart, but which is inserted in a different place in the nuclear genome of the transformed plant than it is in the plant of origin, so that it is not surrounded in the transformed plant by the genes that surround it naturally in the plant of origin.

In principle the heterologous polypeptide synthesized during the malting process may be any polypeptide which may facilitate due to its biological and/or enzymatic activities the degradation or conversion of substances present in the transgenic material. The nucleic acid molecule encoding the heterologous polypeptide synthesized during the process according to the invention may be derived from any organism, in particular from bacteria, fungi, animals or plants. It may be a naturally occurring nucleic acid molecule or a molecule which has been genetically modified. It may be a cDNA or genomic DNA isolated from appropriate libraries as well as a chemically synthesized molecule.

In a preferred embodiment of the present invention the heterologous polypeptide is a polypeptide normally not present in plant cells, in particular a polypeptide that has a biological or enzymatic activity naturally not present in such cells.

A general advantage of the process according to the invention is that the expression of the heterologous polypeptide does

normally not occur during cultivation of the transgenic plants in the field but is specifically induced during the malting process, namely during germination of the malted material and/or during the subsequent development of the embryo. Therefore, the heterologous polypeptide is only produced in the transgenic plant material during the malting process in closed vessels which allows a tight control of expression and thus, provides better safety conditions.

Further advantages are relatively low production costs and the possibility to deliberately vary the amount of processed material (several kilograms up to several tons). A further important feature of the process according to the invention is that it provides a high level of safety in comparison to the production of heterologous products in transgenic plants in planta, since it is carried out in closed vessels, is highly regulated by human intervention and is carried out by using non-pathogenic, harmless material.

According to the present invention the synthesized heterologous polypeptide is an enzyme which leads to the conversion of a storage substance present in the transgenic plant material into a desired product. Said enzyme is set free from the transgenic material and used to act directly on the storage substance in the malting solution.

In a preferred embodiment the degradation and/or conversion of the storage substance leads to the increased production of a substance normally present in plant cells. Examples of such substances are amino acids, fatty acids, lipids, proteins, peptides, polyphenols, steroids, alkaloids, plant hormones, carbohydrates such as polysaccharides, monosaccharides or oligosaccharides, secondary metabolites, especially those which accumulate in plants only in very low concentrations and which may have medical applications, etc.

Further examples are sugar alcohols like mannitol, pinitol or ononitol. Enzymes catalysing the synthesis of these sugar alcohols are, for example, the mannitol-1-phosphate dehydrogenase encoded by the mtlD gene of E. coli or the myo-inositol-O-methyl transferase encoded by the imt gene of Mesembryanthemum crystallinum. Yet another example is trehalose.

In yet another preferred embodiment the degradation and/or conversion of the storage substance leads to the synthesis of a substance or a metabolic intermediate normally not present in plants. Examples for such substances are substances categorised as "renewable resources" such as bioplastics, for example, polyhydroxyalkanoate (PHA) or polyhydroxybutyrate (PHB) which normally do not occur in plants but are synthesized in bacteria as storage substances, for example, in Alcaligenes eutrophus. These substances are in particular interesting since they resemble synthetic thermoplastics and display the advantage that they are biodegradable. The genes responsible for PHB synthesis in bacteria have been isolated and published and may be genetically modified and introduced into plant cells (see, for instance, Poirier et al., FEMS Microbiol. Rev. 103 (1992), 237-246; Nawrath et al., Mol. Breeding 1 (1995), 105-122).
Another example are polyfructans which occur mainly as storage substances in bacteria, such as Bacillus species or Erwinia amylophora. DNA sequences coding for enzymes which catalyse the synthesis of polyfructans, such as levan sucrases, are known in the art.

According to the invention it is possible to express heterologous polypeptides which lead to the synthesis of other non-plant substances like saccharides (e.g. cyclodextrins) or fatty acids normally not synthesized in plant cells.

The process according to the invention makes use of the substantial amounts of storage substances normally present in

seeds. Approaches to produce various products in transgenic plants in planta by overexpression appropriate DNA sequences often result in drastic retardation in plant growth since the synthesis of the desired product often significantly interferes with normal plant metabolism. In the case of PHB synthesis, where this problem also occurred even when it was tried to effect PHB synthesis only in the seeds of a plant, this may result from the shuttling of important metabolic intermediates, namely of acetyl-CoA, into the PHB synthesis. These compounds will then not be available for other essential metabolic pathways. The expression of the genes leading to PBH synthesis during the germination in the malting process helps to circumvent these problems. In all known plants of agricultural interest, such as the usual crop plants, the amount of storage substances present in the seed normally exceeds by far the amount required for the first days of development of the seedling after germination. Thus, the storage substances can be easily used during germination and/or during the subsequent development of the seedling to produce other desired substances without significantly interfering with the metabolism of the developing seedling.

Furthermore, the process of germination is a highly regulated process occurring during a short period of time in which due to the catabolic conversion of storage substances extremely high amounts of metabolic intermediates are available. With respect to the possible production of PHB in germinating transgenic material during a malting process, for example, in transgenic seeds of oilseed rape, germination provides for extremely high levels of acetyl-CoA due to the breakdown of the storage substance oleic acid into sugars in the glyoxysomes. Since this is the appropriate substrate for PHB synthesis it is preferable to use transgenic oilseed rape in which the proteins responsible for PHB synthesis are located in the glyoxysomes. Signal sequences providing for localisation in this compartment are known, for example, from

Zhang et al. (Plant Physiol. 104 (1994), 857-864).

According to the invention the nucleic acid sequence which codes for the polypeptide having the desired enzymatic activity is placed under the control of regulatory elements which allow for the expression of the nucleic acid molecule in the transgenic plant material during the malting process. By "regulatory elements" are meant regions of a nucleic acid molecule which regulate expression of a nucleic acid sequence. Such elements may encompass promoters, enhancers, translational enhancers, ribosome binding sites, etc. and optionally poly-A signals. In principle any regulatory element which is functional in plant cells and which is active under the conditions of the malting process according to the invention may be used. The regulatory elements may be homologous or heterologous with respect to the used transgenic plant material and with respect to the nucleic acid molecule encoding the heterologous polypeptide. A list of suitable plant promoters is given, for example, in Nover (Ed.), "Plant Promoters and Transcription Factors", Springer Verlag 1994, Germany. Promoters which direct constitutive expression are, for example, the 35 S promoter of CaMV (Odell et al., Nature 313 (1985), 810-812) or polyubiquitin promoters as those of the polyubiquitin genes of maize (Plant. Mol. Biol. 18 (1992), 675-689). An example for a promoter which is especially useful for the expression in monocotyledonous plants, namely in cereal cells, is the pEmu promoter (Last et al., Theor. Appl. Genet. 81 (1991), 581-588).

In a preferred embodiment the regulatory elements are elements which allow expression specifically in the plant material used for the malting process according to the invention.

Preferred regulatory elements are those which comprise promoters specifically active in seeds. Such promoters are,

for example, the USP promoter of Vicia faba which directs seed specific expression in Vicia or other plants (Fiedler et al., Plant Mol. Biol. 22 (1993), 669-679; Baumlein et al., Mol. Gen Genet. 225 (1991), 459-467). Promoters known to be specifically active in the endosperm of maize kernels are, for example, the promoters of the zein genes (Pedersen et al., Cell 29 (1982), 1015-1026; Quattrocchio et al., Plant Mol. Biol. 15 (1990), 81-93).

In yet another preferred embodiment the regulatory elements comprise a promoter specifically active during germination of seeds, preferably a promoter which is specifically active in the aleurone layer of germinating seeds of corn plants.

An example for such a promoter are the promoters of α-amylase genes, for instance, of barley (see, for example, Khursheed and Rogers, J. Biol. Chem. 263 (1988) 18953-18960; Jacobson and Close, Plant Mol. Biol. 16 (1991), 713-724) and wheat (Huttly and Baulcombe, EMBO J. 8 (1989), 1907-1913; Baulcombe et al., Mol. Gen. Genet. 209 (1989), 33-40) and rice (Huang et al., Nucl. Acids Res. 18 (1990), 7007-7014) which are induced by giberellic acid specifically occurring during germination. Another promoter specifically active during germination is, for example, the promoter of the isocitrate lyase of rice (Zhang et al., Plant Physiol. 104 (1994), 857-864).

Other examples of promoters which are active during germination of seeds are, for instance, those of the glucanase genes.

Furthermore, Harada et al. (Mol. Gen. Genet. 212 (1988), 466-473) describe the expression of a distinct set of mRNA during germination of Brassica seeds. These include, for example, the transcripts of the genes coding for isocitrate synthase and malate synthase. The promoters of these genes can be

isolated according to conventional techniques known to those skilled in the art from genomic libraries, for example, from oilseed rape or from sunflower.

Furthermore, regulatory elements may be used which are inducible by external influences and thereby allow the exact control of the expression of the heterologous polypeptide of interest. Examples for such regulatory elements are promoters which are inducible by specific chemical or physical influences. Of particular interest are promoters of heat shock proteins which may be easily activated by an increase in temperature during the malting process. Other examples are wound inducible promoters (Velten et al., EMBO J. 3 (1984), 2723-2730).

According to the invention it is possible that the synthesized heterologous polypeptide is either located in the transgenic plant cells or is transported to the apoplast. If the polypeptide is located in the cell, it may be located in any suitable compartment, such as the cytoplasm, the plastides, the vacuole, the mitochondria or the endoplasmatic reticulum. Signal sequences or leader peptides which ensure the location of proteins in the above mentioned compartments of plant cells are known in the art and the corresponding DNA sequences can be fused to the coding region of the heterologous polypeptide of interest by the skilled person according to well known techniques. The location of the heterologous polypeptide in the apoplast of the transgenic material may be achieved by fusing to the coding region of the polypeptide a signal sequence leading to transport in the apoplast. Such sequences are also known in the art, for example, from apoplastic invertase of potato. The location in the apoplast may be desired if it is intended that the heterologous protein acts on a substance present outside the transgenic material, namely in the malt solution.

In the case that nucleic acid sequences are used which are not derived from plants, it might be necessary to modify these sequences in order to obtain efficient expression in plant cells. This applies especially to sequences derived from microorganisms, for example from bacteria. This can be achieved, for example, by adapting the GC-content of the sequence to that usually occurring in the transgenic plant material used for the process according to the invention. It is known, for instance, that most of the monocotyledonous plants display in coding regions specifically expressed during germination a preference for the bases guanine and cytosine (Campbell and Gowri, Plant Physiol. 92 (1989), 1-11; Murray et al., Nucl. Acids Res. 17 (1989), 477-498). In contrast thereto some bacterial genes display no preference but show rather an equal distribution of all four bases. The possibility to improve expression of a bacterial gene by adapting the sequence to the preferred codon usage of plants is described, for example, by Perlak et al. (Proc. Natl. Acad. Sci. USA 88 (1991), 3324-3328) for a Bacillus insecticide.

According to the invention it is not only possible to express one polypetide during the malting process in the used transgenic plant material, but it is also possible to express two or more heterologous polypeptides simultaneously or in succession. This may be useful if these polypeptides are enzymes involved in the same catabolic or metabolic pathway or if the product of the catalytic reaction of one heterologous polypeptide is the substrate for the catalytic reaction of another heterologous polypeptide. Thus, it is also possible according to the invention to use transgenic plant material comprising several nucleic acid sequences coding for heterologous polypeptides which act in concert in the degradation or synthesis of substances in plant cell. Examples would be coding sequences from bacterial operons encoding proteins which catalyse a biochemical pathway not

present in plant cells, for example, PHA or PHB synthesis.

As a result of the process according to the invention one or more heterologous polypeptides are synthesized which may be present in the malted transgenic plant material and/or in the malting solution and which lead, due to their biological and/or enzymatic activity, to the production of a desired degradation and/or conversion product of the storage substance. Said degradation and/or conversion product present in the malted material or the malting solution can be isolated from said material by methods known in the art. The malted transgenic material and/or the malting solution may be useful for different purposes.

Thus, the present invention also relates to the malted transgenic material as well as to the malting solution obtainable by the process according to the invention, whereby the malted material or malted solution may be used as such or after partial purification and/or combination with other known compounds for different purposes in various kinds of fields.

Hence, the present invention also relates to compositions comprising the malted transgenic plant material or the malting solution obtainable by the process according to the solution in raw or purified form. Depending on the nature of the heterologous polypeptide synthesized in the malted material and/or solution, the composition may be useful, for example, as a nutrient or as a pharmaceutical composition. Other possible applications are in the chemical industry as analytical means or catalysts or as additives, for example, in non-food or food sector. Also the use as diagnostic tools is possible.

In a preferred embodiment the pharmaceutical composition according to the invention is useful in therapeutic applications or in prophylactic applications. Of particular interest

is, for example, the possible use for vaccination by oral application.

The construction and introduction of the recombinant nucleic acid molecule which is present in the transgenic plant material can be carried out according to methods known in the art. These methods comprise, for example, techniques for the preparation, manipulation, characterization and cloning of nucleic acid molecules as described, for instance, in Sambrook et al. (Molecular Cloning, A Laboratory Manual, 2nd Ed. (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.).

For the introduction of foreign DNA into higher plants there exists already a plurality of suitable transformation methods and vectors which allow for the integration of foreign DNA into the plant cell genome.

Normally the DNA used for transformation further contains a DNA coding for a selectable marker in order to facilitate the identification and propagation of transformed cells. Examples for marker genes are those encoding proteins that can confer resistance against an antibiotic, such as Kanamycin, Hygromycin, G418 or Bleomycin. Other preferred marker DNAs code for proteins that can provide a distinguishable colour to the plant cell, such as the A1 gene encoding dihydroquercetin-4-reductase (Meyer et al., Nature 330 (1987), 677-678) and the glucoronidase gene (Jefferson et al., Proc. Natl. Acad. Sci. USA 83 (1988), 8447) or that confer to the transgenic plant a characteristic morphological feature such as dwarf growth or a different shape of leaves. Other examples are marker DNAs which confer improved stress tolerance or disease or pest resistance, for example DNAs coding for endotoxins of Bacillus thuringiensis. Further examples of markers are those conferring resistance to herbicides by inhibiting or neutralising their action, such as bar gene, the sfr gene and the

sfrv gene which confer resistance to glutamine synthetase inhibitors such as bialaphos. Possible is also the utilisation of marker DNAs which encode modified target enzymes that have a lower affinity for a specific herbicide, such as a modified glutamine synthetase resistant to phosphinotricine. Other examples are marker DNAs encoding proteins which neutralise the action of the herbicide bromoxynil (Stalker et al. (1988), in: Genetic Improvements of Agriculturally Important Crops, Ed: Fraley, Frey and Schell, Cold Spring Harbor Laboratories).

Also vital markers like the "green fluorescent protein" (Sheen et al., Plant J. 8 (1995), 777-784) may be used.

Methods for the integration of foreign DNA into plant cells include, for example, the transformation of plant cells or of plant tissue with T-DNA using Agrobacterium tumefaciens or Agrobacterium rhizogenes as transformants. These methods have been extensively examined and described in detail (see, for example, EP-A 116 718; EP-A 120 516; Hoekema, In: The Binary Plant Vector System, Offsetdrukkerij Kanters B.V., Ablasserdam, Chapter V; Fraley et al., Crit. Rev. Plant. Sci. 4, 1-46 and An et al., EMBO J. 4 (1985), 277-287).

Other methods are the direct gene transfer as described, for example, in EP-B 164 575, the fusion of protoplasts, injection, electroporation of DNA, the introduction of DNA via the biolistic technique.

In general, the transformation of dicotyledonous plants is well established. Beside transformation protocols for most of the agriculturally important species like potato, transformation protocols have also been established for sunflower (see, for example, Knittel et al., Plant Cell Reports 14 (1994), 81-86) and for oilseed rape (Fry et al., Plant Cell Reports 6 (1987), 321-325). Furthermore, recent developments

also allow the transformation of various monocotyledonous plants via different transformation techniques. Some monocotyledonous plants are susceptible to Agrobacterium infection and had been shown to be transformable via this method (see, for instance, Deng et al., Science in China 33(1990), 28-34; Wilmink et al., Plant Cell Reports 11 (1992), 76-80; May et al., Bio/Technology 13 (1995), 486-492; Conner and Dommisse, Int. J. Plant Sci. 153 (1992), 550-555; Ritchie et al., Transgenic Res. 2 (1993), 252-265; Hiei et al., Plant J. 6 (1994), 271-282).

An alternative method for the transformation of monocotyledonous plants is also the biolistic method (see, for example, Wan and Lemaux, Plant Physiol. 104 (1994), 37 48; Vasil et al., Bio/Technology 11 (1993), 1553-1558; Spencer et al., Theor. Appl. Genet. 79 (1990), 625-631; Ritala et al., Plant Mol. Biol. 24 (1994), 317-325). Described are also methods which are based on the transformation of monocotyledonous protoplasts, the electroporation of partially permeabilised cells or the introduction with the help of glass fibres.

Documents describing, for example, the specific transformation of maize are EP-A 513 849, EP-A 465 875 and WO 95/06128 as well as Fromm et al. (Biotechnology 8 (1990), 833-844), Gordon-Kamm et al. (Plant Cell 2 (1990), 603-618) and Koziel et al. (Biotechnology 11 (1993), 194-200). Also the regeneration of maize plants from different starting materials has been described (see, for instance, EP-A 292 435, Shillito et al., Bio/Technology 7 (1989), 581 and Prioli and Söndahl, Bio/Technology 7 (1989), 589).

The successful transformation of wheat has been described, for example, in Weeks et al. (Plant Physiol. 102 (1993), 1077-1084), Nehra et al. (Plant J. 5 (1994),285-297) or Becker et al. (Plant J. 5 (1994), 299-307) as well as the transformation of barley (Wan and Lemaux, loc. cit. and Ri-

tala et al., loc. cit.; Ritala et al., Euphytica 85 (1995), 81-88; Harwood et al., Euphytica 85 (1995), 113-118; Funatsuki et al., Theor. Appl. Genet. 91 (1995), 707-712; EP-A 0 483 847).

The production of transgenic rice plants has been described, for example, in Christou et al. (Biotechnology 9 (1991), 957-962) and Peng et al. (Theor. Appl. Genet. 83 (1992), 855-863) and Somers et al. (Biotechnology 10 (1992), 1589-1594) describes the transformation and regeneration of fertile rye plants. For a review of rice transformation, see also Ayres and Park, Crit. Rev. Plant Sci. 13 (1994), 219 239.

Thus, monocotyledonous plants as well as dicotyledonous plants may be genetically modified according to techniques known in the art to obtain plants which may be used as a source for the transgenic material employed in the process according to the invention.

Figure 1 shows schematically the structure of plasmid pEmuBmCGTase used for the transient expression in transformed protoplasts. In this construct the coding region of the gene coding for CGTase from Bacillus macerans is fused to the pEmu promoter as described by Last et al. (loc. cit.). ARE and OCS are enhancer elements; dADH is the promoter of the Adh gene from maize and Adh1 Intr1 stands for the first intron of the Adh gene. Nos stands for the termination signal of the nopalin synthase gene of Agrobacterium tumefaciens.

Figure 2 shows schematically the structure of plasmid pEmuKpCGTase used for the transient expression in transformed protoplasts. In this construct the coding region of the gene coding for CGTase from Xlebsiella pneumonieae is fused to the pEmu promoter as described by Last et al. (loc. cit.). For the abbreviations see Figure 1.

Figure 3 shows schematically the structure of plasmid pAmyBmCGTase used for transformation of wheat and barley. In this construct the coding region of the gene coding for CGTase from Bacillus macerans is fused to the signal sequence of the α-amylase and to the promoter of the amylase high pI gene. E9 trm stands for the transcriptional termination sequence E9.

Figure 4 shows schematically the structure of plasmid pAmyKpCGTase used for transformation of wheat and barley. In this construct the coding region of the gene coding for CGTase from Klebsiella pneumonieae is fused to the signal sequence of the α-amylase and to the promoter of the α-amylase gene.

The examples illustrate the invention.

Example 1

Construction of plant expressible CGTase-Genes

In order to achieve synthesis of cyclodextrines in plants, DNA sequences coding for cyclodextrin glycosyltransferases (CGTases) are isolated from Klebsiella pneumoniae (Binder et al., (1986) loc. cit.) and from Bacillus macerans (Takano et al., loc. cit.) via PCR technique (Saiki et al., Science 239 (1988), 487-491). The amplified coding sequences are cloned into vectors containing either the α-amylase promoter of barley (Khursheed and Rogers, J. Biol. Chem. 263 (1988) 18953-18960) and the nos terminator or the polyubiquitin promoter of maize (Christensen et al., Plant Mol. Biol. 18 (1992), 675-689) or the pEmu promoter, based on a truncated maize Adh1 promoter (Last et al., loc. cit.) and the nos terminator. For the construction of vectors which ensure secretion of the CGTase from the aleuroplast, the coding sequence of mature CGTase was cloned behind the signal sequence

of α-amylase. Figures 1 to 4 "cinematically show the structure of the constructed cloning vectors comprising the expression cassettes.

Recombinant plasmids in which the coding sequence is linked in sense-orientation to the promoter are selected. From these the expression cassettes are isolated by restriction digest and inserted into suitable plant transformation vectors. These vectors carried also a uid-A gene coding for GUS (Jefferson et al., (1987) loc. cit.) and a bar gene, which confers resistance to the herbicide bialaphos.

Example 2

Transient expression of CGTase genes in plant protoplasts

In order to prove the expression of the chimeric CGTase genes in plant cells, aleuron cell protoplasts are prepared from wheat and barley (Lee et al., Plant Mol. Biol. 13 (1989), 1-29) and transfected with the transformation vectors described in Example 1 which contain the maize polyubiquitin promoter in the presence of polyethylene glycol (Chand et al., J. Plant Physiol. (1988), 480-485). In order to check whether any active cyclodextrin glycosyltransferases are synthesized, the transformed protoplasts are cultivated for 36 hours in induction medium. The cell free medium is then mixed with starch (7% final concentration) and incubated for 12-24 at room temperature. The medium is passed through a C18 Seppak column (Millipore) in order to remove starch and recovered cyclodextrins are analysed; see infra.

Example 3

Transformation of barley and wheat and analysis of the regenerated plants

The plant transformation vectors described in Example 1 containing the barley α-amylase promoter are used to transform wheat and barley immature zygotic embryos, young callus or microspore-derived embryos according to the method described in Wan and Lemaux (loc. cit.). For this purpose grains of wheat and barley plants are germinated under conditions described by Wan and Lemaux (1994, loc. cit.)and developing plants are kept in the chamber until immature embryos develop. These are subsequently placed on callus medium to induce proliferation and callus formation. The developing calli are several times divided, removed and placed onto new medium before the cell material is competent for transfomation via particle bombardment. The bombardment is carried out as described by (Weeks et al., (1993), loc. cit.; Nehra at al., (1994), loc. cit.; Wan and Lemaux, (1994), loc. cit.; Becker et al., (1994), loc. cit.; or Ritala et al., (1994), loc. cit.).

After bombardment the embryos or callus material is transferred to culture plates containing regeneration medium containing bialaphos (Spencer et al., Theor. Appl. Genet. 97 (1990), 625-631) to select for transformed cells. Bialaphos resistant callus tissue is further cultivated and after regeneration of plants transferred to hormone free plant medium (Wan and Lemaux, (1994), loc. cit.).

Example 4

Genetic analysis of transformed plants

In order to verify that the CGTase sequence is present in the transformed plants nuclear DNA is prepared from leaf tissue of transformed plants according to standard techniques. The DNA is digested with a set of restriction enzymes and separated on agarose gels. The restriction fragments are transferred to a nylon membrane and hybridised with labelled the CGTase gene fragment according to Chowdhury et al. (Theor.

Appl. Genet. 87 (1994), 821-828) and Wan and Lemaux (1994, loc. cit.). Alternatively the presence of the CGTase coding sequence is verified by PCR technique using suitable oligonucleotide primers.

Transformed plants which harbour the CGTase coding sequence are placed in climate chambers in order to allow regeneration of fully developed and fertile plants.

The stable integration of the CGTase gene into the genome of the primary transformant is subsequently demonstrated by the analysis of the progeny plants obtained by selfing and by backcrossing.

Example 5

Detection of cyclodextrin production in transformed plants

For the expression analysis of functional CGTases in transgenic wheat and barley plants are cultivated in climate chambers until they set seeds. The grains are harvested and induced to germinate. Soluble polysaccharides are extracted as described by Oakes et al. (Biotechnology 9 (1991), 982-986). For the quantification of cyclodextrin production the conversion of soluble starch in cyclodextrins is determined by measuring in parallel the remaining starch content (Keeling et at., Planta 191 (1993),342-348), the insoluble and soluble starch fractions (Murugesan et al., Carbohydr. Res. 242 (1993), 203-215) and the ratio of amylose/amylopectin (Hovenkamp-Hermelink et al., Potato Res. 31 (1988), 241-246). Cyclodextrins are further analysed by HPLC according to (Koizumi et al., J. Chromatograph. 341 (1985), 31-41; Frijlink et al., J. Chromatogr. 415 (1987), 325-333; Fukuda et al., Anal. Biochem. 212 (1993), 289-291; Oakes et al., Biotechnology 9 (1991), 982-986).

Example 6

Malting of transgenic seeds expressing CGTase

In order to subject the transgenic seeds obtained from transgenic plants which had been obtained according to Example 3 to a malting process and to achieve expression of the CGTase in the germinating seeds the following steps were carried out:

1. Steeping of the seeds until the development of rootless can be observed. This results in the production of the so called chit malt.

2. Transfer of the chit malt to the germination boxes where it is continuously moistened. Temperature, pH value, humidity and length of the process are so controlled that the seeds can germinate and that the CGTase gene is expressed. This results in the production of green malt expressing CGTase.

3. Conversion of the starch present in the green malt into cyclodextrins.

This step was carried out in two different ways:

a) The green malt was incubated at pH 6.0 and at 50 °C, which are the optimal conditions for the CGTase of Bacillus macerans. By controlling the process in an appropriate way it is possible to activate in the first place amylases which lead to the liquidisation of starch and the CGTase which subsequently converts the amylose into cyclodextrins.

The green malt is subsequently kiln dried or dried by microwave treatment. The cyclodextrins can be isolated from the cured malt.

b) Alternatively the conversion of starch into cyclodextrins took place in liquid medium. For this purpose the green malt was broken up mechanically. In this step it is optionally possible to separate the husk from the endosperm. The endosperm was the converted into a mesh by adding water using an agitator. The enzymatic conversion then took place in the liquid medium. Synthesized cyclodextrins can be recovered directly from the mash by appropriate extraction steps, such as precipitation with the help of additives or of chromatography.

Claims

1. A process for the production of degradation and/or conversion products of storage substances present in transgenic plant material, which process comprises

(a) subjecting transgenic plant material to a malting process, wherein the transgenic plant material comprises at least one recombinant nucleic acid molecule containing

(i) at least one nucleic acid sequence coding for at least one heterologous polypeptide possessing enzymatic activity which leads to the degradation and/or conversion of a storage substance present in the transgenic plant material; and

(ii) regulatory elements which allow for the expression of said nucleic acid sequence(s) in the transgenic plant material during the malting process,

whereby said heterologous polypeptide(s) is (are) synthesized; and

(b) degrading and/or converting the storage substance present in the transgenic plant material by said heterologous polypeptides in the malting solution.

2. The process of claim 1 wherein the transgenic plant material is a seed.

3. The process of claim 1 or 2 wherein the transgenic plant material is derived from a monocotyledonous plant.

4. The process of claim 1 or 2 wherein the transgenic plant material is derived from a dicotyledonous plant.

5. The process of any one of claims 1 to 4 wherein the

transgenic plant material is derived from a transgenic plant which is a starch storing plant.

6. The process of claim 5 wherein the starch storing plant is a corn plant.

7. The process of claim 6 wherein the corn plant is wheat, barley, oats, rye, rice, maize or millet.

8. The process of claim 5 wherein the starch storing plant is a leguminous plant.

9. The process of claim 8 wherein the leguminous plant is pea or beans.

10. The process of anyone of claims 5 to 9 wherein the enzymatic activity is the enzymatic activity of an amylase, a branching enzyme, a debranching enzyme, a disproportioning enzyme, a starch phosphorylase, a dextrinase or a cyclodextrin glycosyltransferase.

11. The process of claim 10 wherein the cyclodextrin glycosyltransferase is derived from a Bacillus species or from Klebsiella pneumonia.

12. The process of any one of claims 1 to 4 wherein the transgenic plant material is derived from a transgenic plant which uses fats or oils as a storage substance.

13. The process of claim 12 wherein the transgenic plant is rapeseed or sunflower.

14. The process of any one of claims 1 to 4 wherein the transgenic plant material is derived from a transgenic plant which uses proteins as a storage substance.

15. The process of claim 14 wherein the transgenic plant is soybean.

16. The process of claim 14 or 15 wherein the enzymatic activity is the enzymatic activity of a protease and/or of another protein-modifying enzyme.

17. The process of any one of claims 1 to 13 wherein the heterologous polypeptide is of bacterial, fungal, animal or plant origin.

18. The process of any one of claims 1 to 17 wherein the degradation and/or conversion leads to the production of a substance or metabolic intermediate normally present in plant cells.

19. The process of claim 18 wherein the substance or metabolic intermediate is an amino acid, a carbohydrate, a fatty acid, a lipid, a polyphenol, a plant hormone or a steroid.

20. The process of any one of claims 1 to 17 wherein the degradation and/or conversion leads to the production of a substance or metabolic intermediate normally not present in plant cells.

21. The process of claim 20 wherein the substance is polyhydroxyalkanoate, polyhydroxybutyrate or poly fructose.

22. The process of any one of claim 1 to 21 wherein the regulatory elements specifically direct expression in the tranagenic plant material used for the process.

23. The process of claim 22 wherein the regulatory elements comprise a seed specific promoter.

24. The process of claim 23 wherein the seed specific promoter specifically directs expression during germination.

25. The process of claim 23 or 24 wherein the promoter is an aleurone specific promoter.

26. The process of claim 25 wherein the promoter is the promoter of an a-amylase gene.

27. The process of claims 22 or 23 wherein the regulatory elements comprise an inducible promoter.

28. The process of any one of claims 1 to 27 wherein the synthesized heterologous polypeptide is located in the cells of the transgenic plant material.

29. The process of any one claims one 1 to 27 wherein the synthesised heterologous polypeptide is located in the apoplast of the transgenic plant material.

30. The process of any one of claims 1 to 29 wherein the process further comprises

(c) isolating the degradation and/or conversion product.

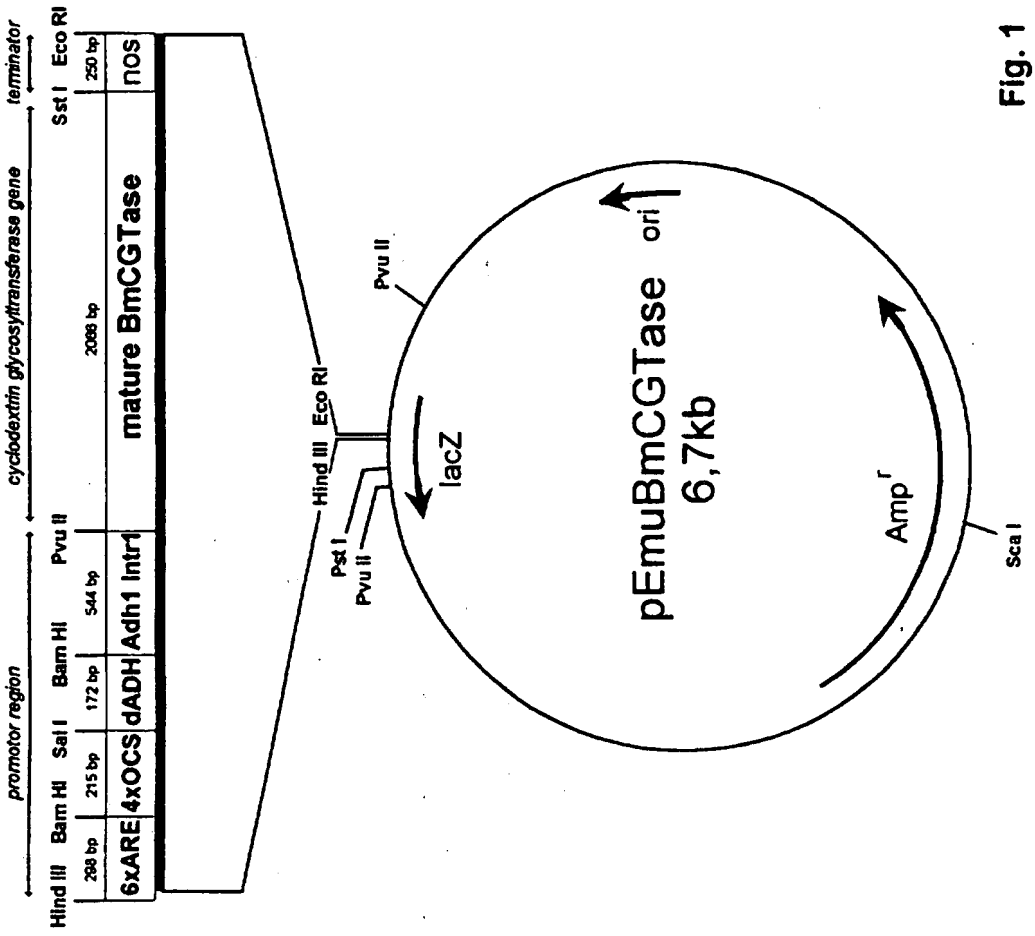
31. Malted transgenic plant material and/or malting solutions obtainable by a process of any one of claims 1 to 29.

32. A composition comprising malted transgenic plant material and/or malting solution of claim 31.

33. The composition of claim 32 which is useful as a nutrient.

34. The composition of claim 32 which is useful as a pharmaceutical, therapeutic or prophilactic composition.

35. The composition of claim 34 which is useful as a vaccine for oral application.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544 bp
2088 bp
250 bp
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4xOCS
dADH
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Fig. 1

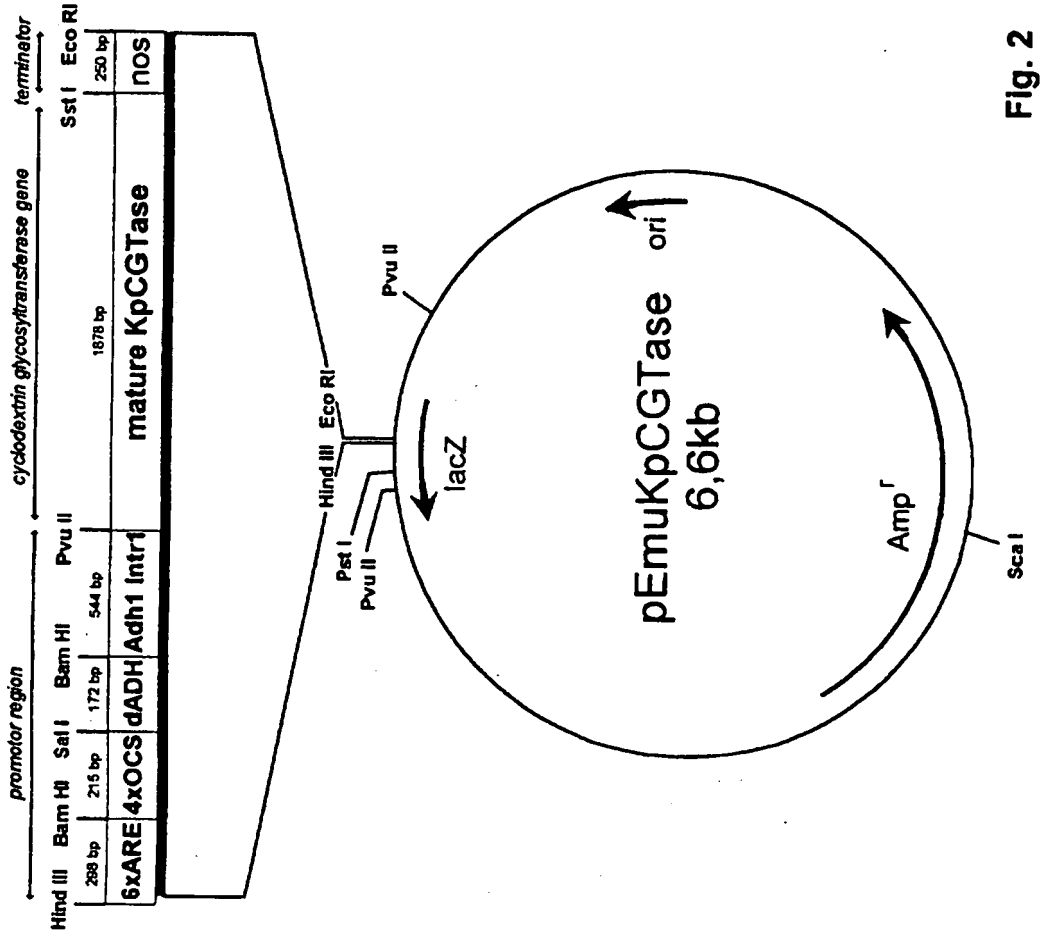
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Fig. 2

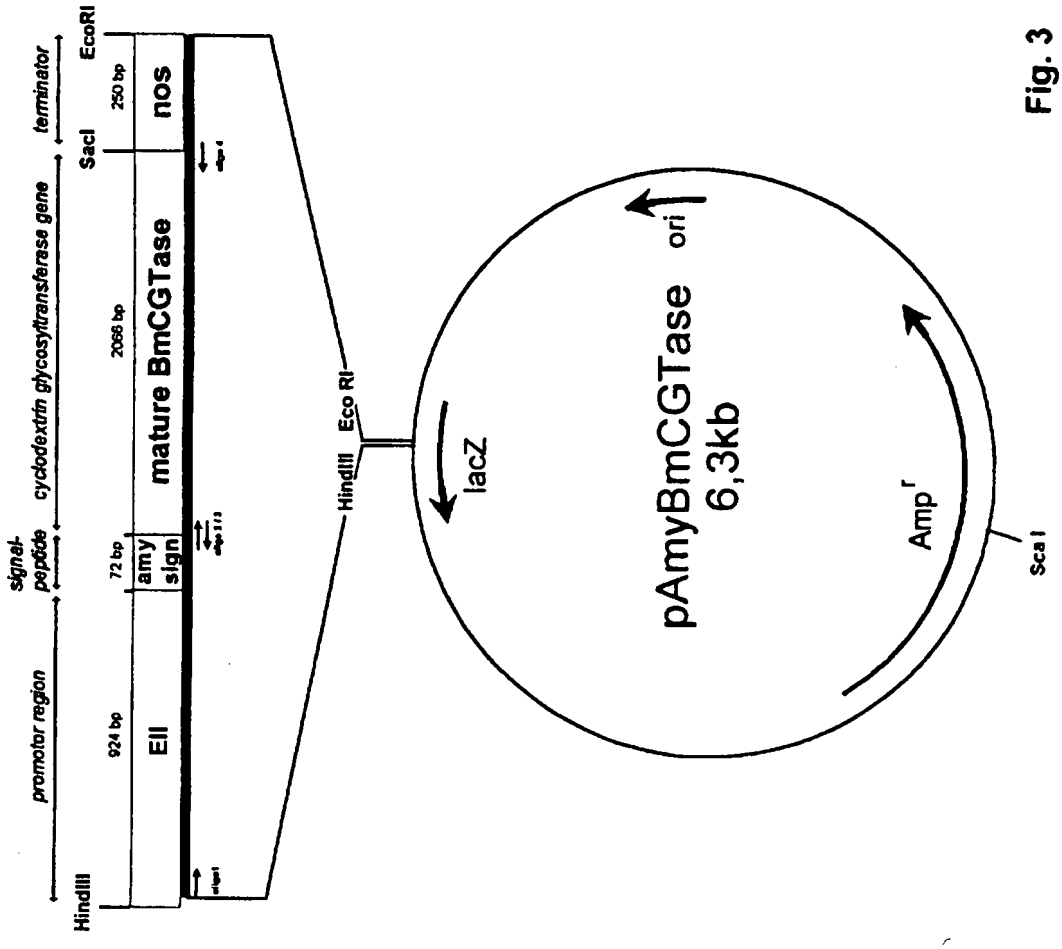
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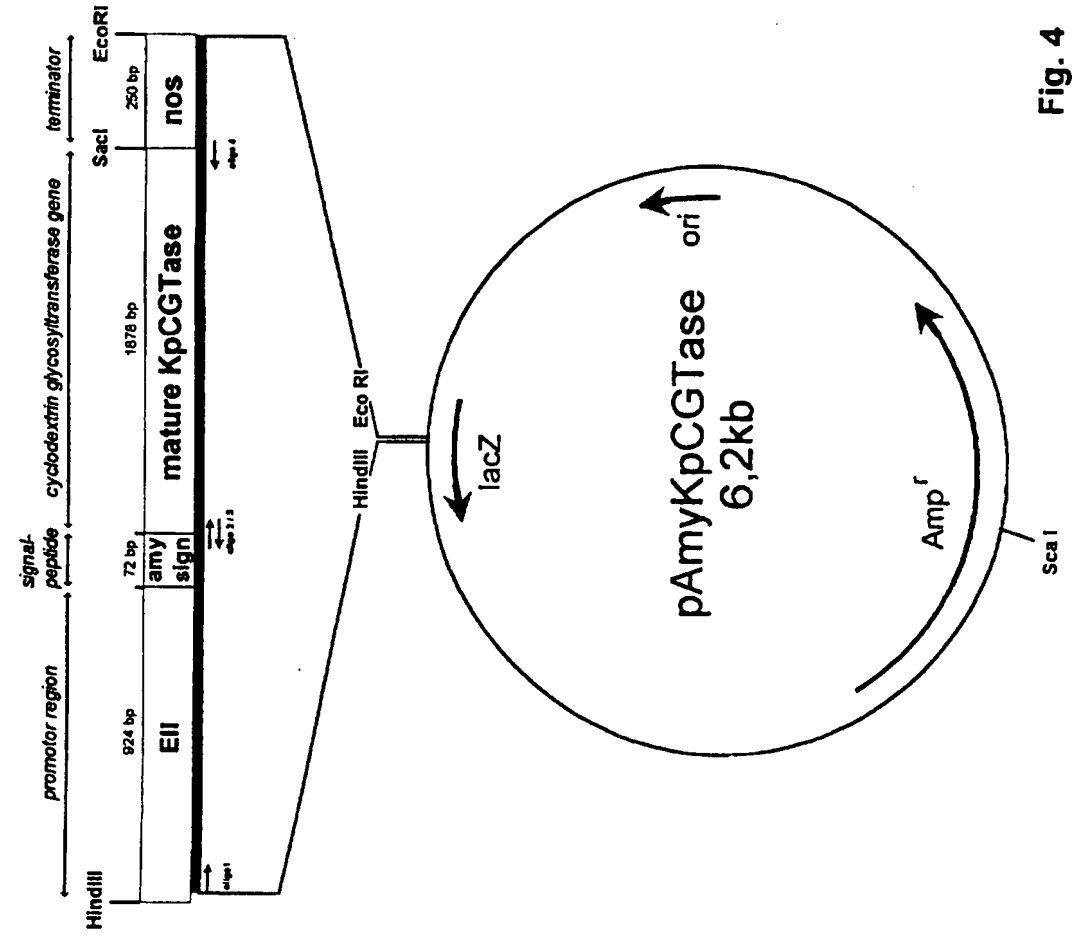
Fig. 3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terminator
250 bp
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lacZ
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Fig. 4